

Comparison of the efficacy of carboplatin, oxaliplatin and cisplatin, with
various concentrations of epidermal growth factor family ligands, in
killing human triple-negative breast cancer cells

An Undergraduate Honors Thesis

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Abstract

Current breast cancer therapies focus on the use of hormones to control tumor cells. However, triple negative breast cancers (TNBC) lack receptors for the hormones estrogen and progesterone, in addition to the HER-2 protein. As such, TNBC therapy is often limited to non-targeted chemotherapeutic agents. Another receptor present on the cell surface of most TNBC cells is epidermal growth factor receptor (EGFR), which could serve as a target for new therapies. EGFR drives cell proliferation following its activation by epidermal growth factor (EGF) family ligands. These ligands include amphiregulin, betacellulin, and EGF, which were chosen for use in this study. Little research has been done on the use of EGF family ligands in combination with established cancer therapies. EGF has been shown to have an effect on the rate of cancer cell death when combined with platinum-based DNA alkylating agents in head and neck cancers. In this combination therapy, the conflicting messages from the stimulating ligand and the alkylating agent cause an enhanced rate of cell death apparently by more targeted and efficient drug delivery to the cell nucleus. We have hypothesized that EGF family ligands can be combined with alkylating agents for synergistic killing of EGFR-expressing TNBC cells. The tests of hypothesis described here were pharmacological dose-response studies. Our results show that different EGF family ligands have markedly different effects on EGF receptor trafficking and subsequent cell death when combined with platinum drugs. To extend our results beyond breast cancer cells, the treatments also were compared in another EGFR-expressing cancer model, brain glioblastoma.

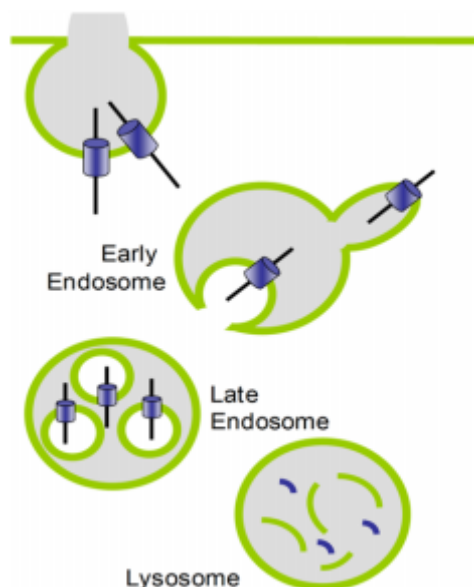
Introduction

Many advances in the treatment of breast cancer have occurred over the past 20 years. An increase in the early detection of the disease, more sensitive screening in regards to tumor type, and refined surgical procedures have all combined to reduce greatly the mortality risk associated with the disease. One of the most significant advances in the treatment of breast cancer is with adjuvant hormone treatment, including drugs such as tamoxifen, which acts as an estrogen receptor antagonist.

There exist, however, hormone-negative cancers such as triple negative breast cancers (TNBC), which lack receptors for estrogen and progesterone and the HER-2 protein, all common targets of other breast cancer therapies. As such, current treatment regimens targeting these receptors are useless to patients with TNBC. Treatment for these patients is often limited to simple systemic chemotherapy and invasive surgery. This fact, combined with the more aggressive and metastatic nature of TNBC, gives patients a far more negative clinical prognosis.

Another characteristic of aggressive tumors, including TNBC, is the overexpression of epidermal growth factor receptors (EGFR) (1). These receptors, when stimulated by epidermal growth factor (EGF), trigger the cell to move into the growth cycle, and their overexpression is correlated to an increased risk of metastasis and a more negative prognosis (2). EGFR is a plasma membrane protein that stimulates cell growth. When activated, EGFR can be internalized by the cell and degraded in lysosomes along with the signaling ligand. Therefore, treatment by a substance that stimulates EGFR may cause a decrease in EGFR concentration on the cell surface (referred to as downregulation), and with this a decrease in subsequent cell sensitivity to stimulation (3). In addition to EGF, other ligands belonging to the EGF family are able to bind to these receptors and cause receptor activation.

Figure 1. Cycle of EGFR when stimulated. When activated by EGF or another ligand, EGF is internalized and moved into the endosome. At the late endosome phase, different effects of distinct ligands can be observed. In specific cases, the receptors are then degraded in the lysosome.



While binding of EGF results in receptor internalization and degradation that signals cell growth, other EGF family ligands vary in their resulting signals. For example, amphiregulin (AMPHI) will bind to the receptor and cause internalization, but the receptor recycles back to the cell surface. This makes the ligand an ideal control in experiments involving EGFR

downregulation. Betacellulin (BTC) binds tightly to the receptor, stimulating a high degree of growth. However, BTC-stimulated receptors are driven efficiently toward lysosomes for degradation.

Alkylating agents have been used in the treatment of aggressive metastatic cancers for decades. Currently, they are most commonly used to treat late-stage lung, ovarian, and brain cancers. Alkylating agents induce G-C crosslinks in actively replicating DNA, and this irreparable damage leads to cell death. Platinum-based alkylating agents are not commonly used in breast cancer treatments, due to the high levels of resistance to cisplatin-induced toxicity (4). The clinical use of alkylating agents also is limited by their severe side effects and frequent toxicity, including severe anemia, esophageal ulceration, and bone marrow toxicity. It is therefore very important to use alkylating agents at the lowest possible effective dose, in order to minimize adverse effects on the patient while causing significant damage to the tumor.

Relatively little has been published about the use of EGF family ligands with alkylating agents in cancer therapy. Head and neck squamous cell carcinomas (HNSCC) overexpress EGFR; they are more sensitive to alkylating agents when co-treated with EGF (5). Reported data suggest that the targeting of EGFRs into the lysosomal degradation pathway is a critical requirement for EGF-dependent enhancement of cell killing by cisplatin (1). We tested the hypothesis that, when alkylating agents (which damage DNA) are combined with any EGF family ligand that induces lysosomal degradation of EGFR, the two signals tell TNBC cells to stop growing and grow, respectively, at the same time. The conflicting messages thereby enhance cell death. Such a strategy could be useful for eliminating EGFR-positive tumors. This enhanced level of cell death could reduce the amount of alkylating agent needed to cause tumor remission. The growth signal given by the EGF family ligands will drive any cells in the resting phase into the growth cycle, where they can be damaged by alkylating agents. Removing cells from the resting phase at this stage of treatment may lower the rate of future recurrence.

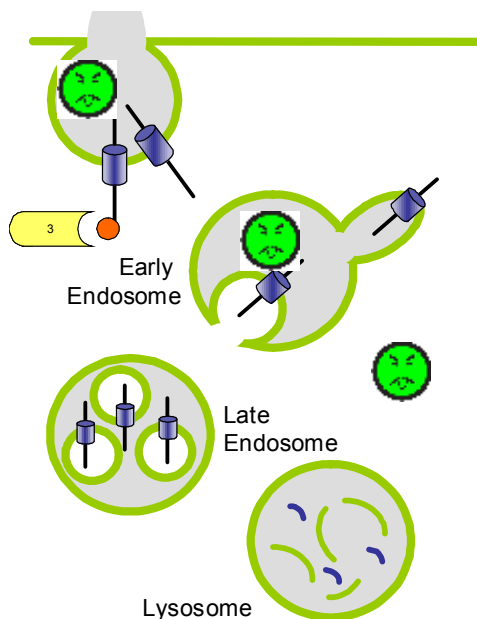


Figure 2. Proposed mechanism of action of drug-ligand combination therapy. EGFR, when stimulated, acts as a “Trojan horse” in delivering the chemotherapeutic drug to the cell. The activated EGFR helps to internalize the alkylating agent, delivering it to the cell nucleus more efficiently than if the alkylating agent were added on its own. Since the platinum-based compounds are used to disrupt DNA but have a short active life, quick and effective delivery to the nucleus increases the rate of cell death in cancer cells. Since only the cancer cells are overexpressing EGFR, this allows for greater delivery of the drug specifically to tumor cells.

Methods

Mammalian cell lines, their passage and maintenance.

Three types of mammalian cell lines were utilized in this study. For the TNBC model, MDA-MB-231-luc-D3H1, an immortalized human adenocarcinoma of epithelial morphology transfected with luciferase gene, was used. It was obtained commercially from Caliper Life Sciences. MDA-MB-231 is a cancer cell type that lacks expression of estrogen, progesterone, and HER-2 protein receptors, and overexpresses EGFR. This matches the characteristics of TNBC, and the cell line has been shown to produce tumors *in vivo* in a mouse model (Caliper Biosciences product information sheet, 2009, “Bioware Cell Line MDA-MB-231-luc-D3H1”, www.caliperls.com/assets/018/7634/pdf). The presence of the luciferase gene will aid in imaging studies in future experiments. Two types of glioma cells were kindly provided by Dr. R. Barth for comparison to the breast cancer model. MDA-MB-F98 is a rat glioma cell line, with two distinct sublines: EGFR Wild Type f (Wf) and EGFR Wild Type T5 (WT5). Wf has a high level of EGFR expression, and the receptors can undergo phosphorylation after stimulation with an EGF-family ligand. WT5 has a lower level of EGFR expression than Wf, but its receptors are not efficiently phosphorylated upon ligand addition (personal communication, R. Barth). Preliminary studies in the Lill laboratory indicate that only the WT5 receptors appear to undergo ligand-induced degradation (unpublished).

All cell lines were passaged regularly at subconfluence, using a 0.25%trypsin/EDTA solution and following an established laboratory protocol. The cells were maintained in “10% Complete” DMEM containing 10% fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 units/mL penicillin-streptomycin and 20 mM HEPES, in an atmosphere containing 5% CO₂.

Preparation of solutions used to treat cell lines.

All three cell lines were treated with several different types of alkylating agents and EGFR family ligands. Cisplatin, carboplatin, and oxaliplatin were obtained from Sigma-Aldrich in powdered form. Cisplatin was prepared in physiological saline (150mM NaCl) to generate a 0.1 mM stock and allowed to solubilize for 24 hours before use. Carboplatin and oxaliplatin were prepared in a 10% dextrose solution to make 3mM and 2mM stocks, respectively, and were used immediately. The stock solutions were used at varying dilutions to generate the working concentrations indicated in the results section. Varying concentrations of EGF and AMPHI obtained from Sigma were prepared in “10% complete” DMEM. BTC obtained from Peprotech was prepared in “10% complete” DMEM.

Treatment of cells *in vitro*.

Cells were removed from dishes with trypsin/EDTA solution and seeded in 6-well cell culture plates at a concentration of 1×10^5 cells per 9.4 cm^2 well. Cells were allowed to grow for 24 hours before treatment. At time of treatment, the media in all wells were removed and replaced with the various treatment solutions as determined per experiment. Cells were then allowed to grow undisturbed for a period of 5 days (for the breast cancer cell line) or a period of 6 days (for the glioma cell lines). Cells were then removed from the dishes using trypsin/EDTA and resuspended in “10% complete” DMEM. Cell suspensions were then combined with 0.4% trypan blue solution and live cells were counted using a Biorad TC10 cell counter.

Statistical Analysis.

For the *in vitro* experiments, the mean and standard deviation of three replicate samples were calculated for each treatment type. A significant difference in matched treatment types was found by calculating the p-value using the student's t-test ($\alpha = 0.05$). Analysis of variance

(ANOVA) was used to determine the significant difference between treatment levels. Triplicate experiments were performed in all cases unless otherwise noted.

Results

Generation of cytotoxicity curves

In order to understand if a combination treatment will work more effectively than an alkylating agent alone in killing MDA-MB 231 cells, a baseline needed to be established. We wished to determine the minimal concentration at which cell death was barely detectable for all three of the alkylating agents. By titrating within a broad range initially and then a narrower range as needed, a break point in the curve of cell death with increasing drug concentration should be observed.

The initial drug concentration range was based on published clinical doses. The common clinical dosage for each drug (in mg/m² of body surface area) was calculated in relation to associated blood volume. The drug solution was prepared in the same vehicle in which the drug is delivered clinically (physiological saline [150mM NaCl] for cisplatin or 10% dextrose solution for carboplatin and oxaliplatin). Several dose ranges were titrated before the final level was selected.

Cisplatin, considered the standard drug for these experiments, was titrated first. Cisplatin was titrated within the concentration range of 0.001 to 5 microM. Dosages in this range were applied to the MDA-MB-231 cells, and the surviving cells were quantified and compared to cells treated with the NaCl vehicle only.

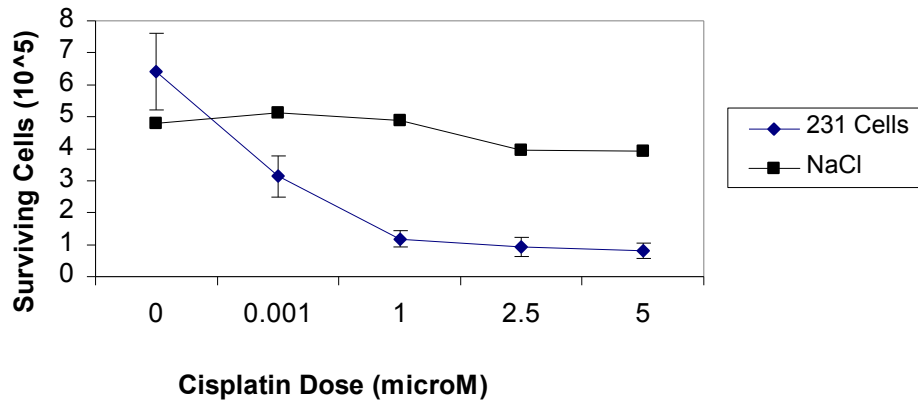


Figure 3. MDA-MB 231 cell survival after 6-day treatment with cisplatin solution. The concentrations of cisplatin used are shown on the x-axis. Averages of three independent experiments and their standard deviations are shown.

The dosage of particular interest to us for future cytotoxicity experiments was the highest dosage at which there was no observable cell death: this dosage is the threshold for determining if a combination treatment will be more effective than an alkylating agent alone. If a significant difference in killing at this drug level is achieved in combination treatment when ligand also is added, the combination treatment can be said to have increased efficacy. Based on my data, the dosage selected for future experiments was .001 microM (Figure 3).

The baseline molar dosages for carboplatin and oxaliplatin were calculated based on the fact that they are considered to be half and one-third as effective as cisplatin, respectively. These drug solutions were prepared in 10% dextrose, as per clinical use instructions. Cytotoxicity curves for these drugs were generated in the same manner as the killing curves for cisplatin. Based on the results of Figures 4 and 5, the baseline dosage for both carboplatin and oxaliplatin is 50 microM.

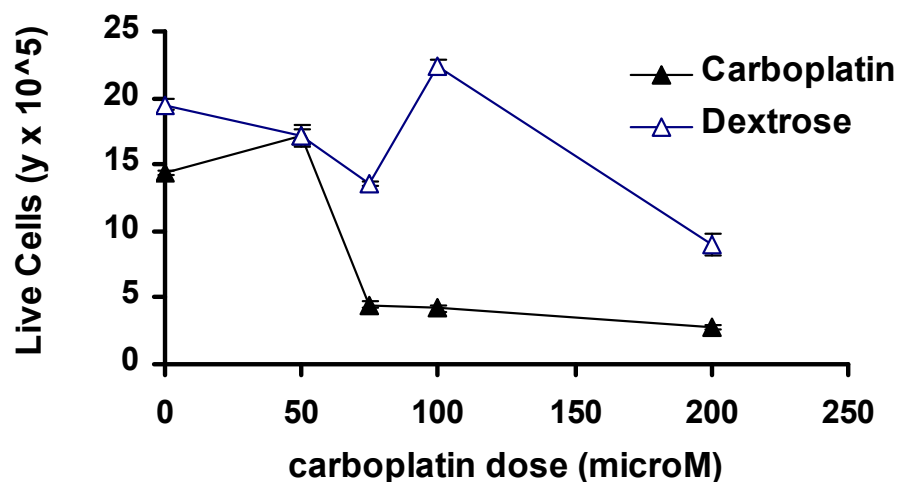


Figure 4. MDA-MB 231 cell survival after 6-day treatment with carboplatin solution. Details of the experimental procedures are provided in the methods section. Averages of three independent experiments and their standard deviations are shown.

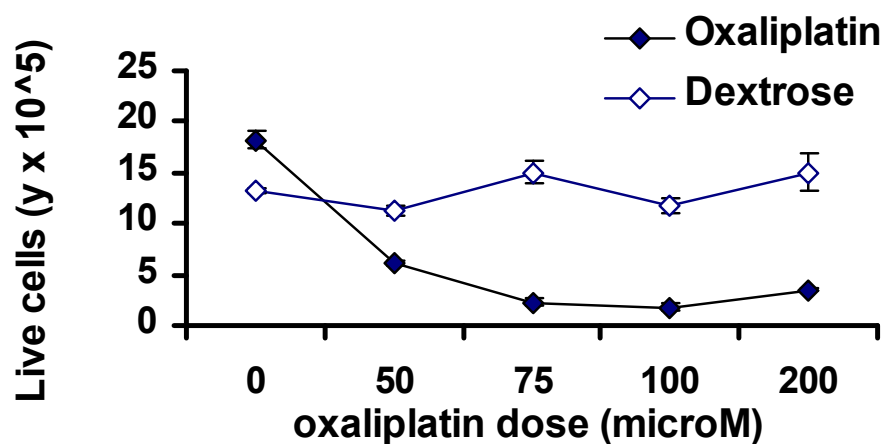


Figure 5. MDA-MB 231 cell survival after 6-day treatment with oxaliplatin solution. Details of the experimental procedures are provided in the methods section. Averages of three independent experiments and their standard deviations are shown.

The glioma cell lines WT5 and Wf were given the same drug dosages as the MDA-MB-231 cells. This produced a similar cytotoxicity curves for these cells, allowing for a comparison between the tumor types (Figure 6).

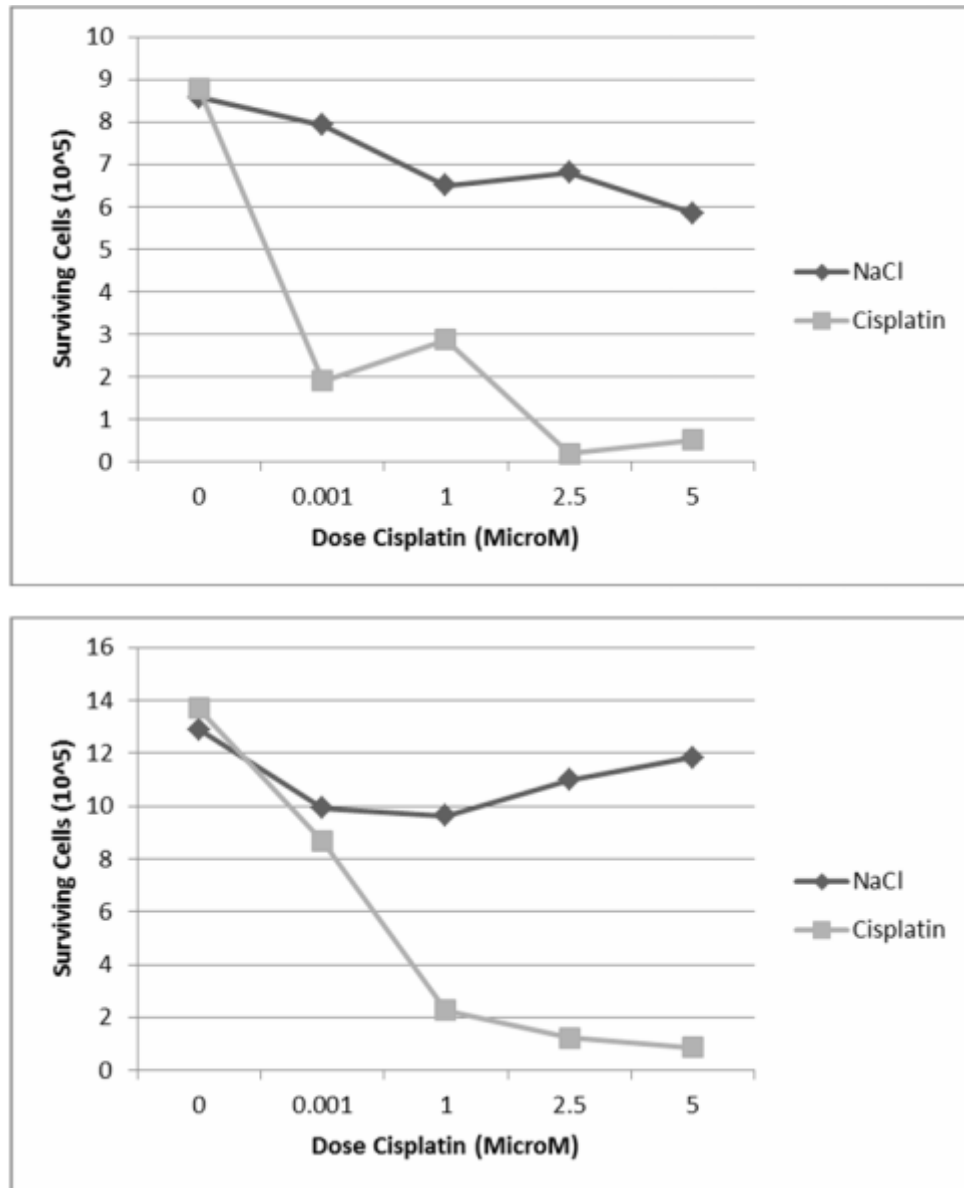


Figure 6. Wf (top) and WT5 (bottom) cell survival after 7-day treatment with cisplatin solution. Details of the experimental procedures are provided in the methods section. Averages of three independent experiments and their standard deviations are shown.

Once the useful baseline dosage of each alkylating agent had been determined, the ligand EGF could be incorporated into controlled experiments to determine if there was a synergistic increase in the levels of cell death. Different working concentrations of EGF were compared for their effects on cell killing by each platinum drug.

For the comparisons in efficacy, the dosage of alkylating agent was held constant. However, whether cells were incubated without or with cisplatin, EGF was titrated into the cultures at increasing doses. The purpose here was to determine whether increasing levels of EGF increasingly cooperated with the selected dose of alkylating agent. We predicted that, in cells receiving no cisplatin, that increasing doses of EGF would have no impact on the number of cells in each culture after 6 days of incubation: the cells receive all necessary amounts of EGF from their culture media, which contains 10% serum. Additional EGF is expected to have no further impact on total cell number. However, for cells receiving cisplatin with increasing doses of EGF, we predicted that with increasing doses of ligand, more cisplatin would be delivered to the cells resulting in enhanced cell death.

For cisplatin in MDA-MB 231 cells, increasing the dose of EGF increasingly enhanced cell killing (Figure 7). The treatment showed no increased efficacy after the dosage of ligand reached the level of 30 microM. This pattern was repeated in the carboplatin and oxaliplatin treated samples (Figure 8).

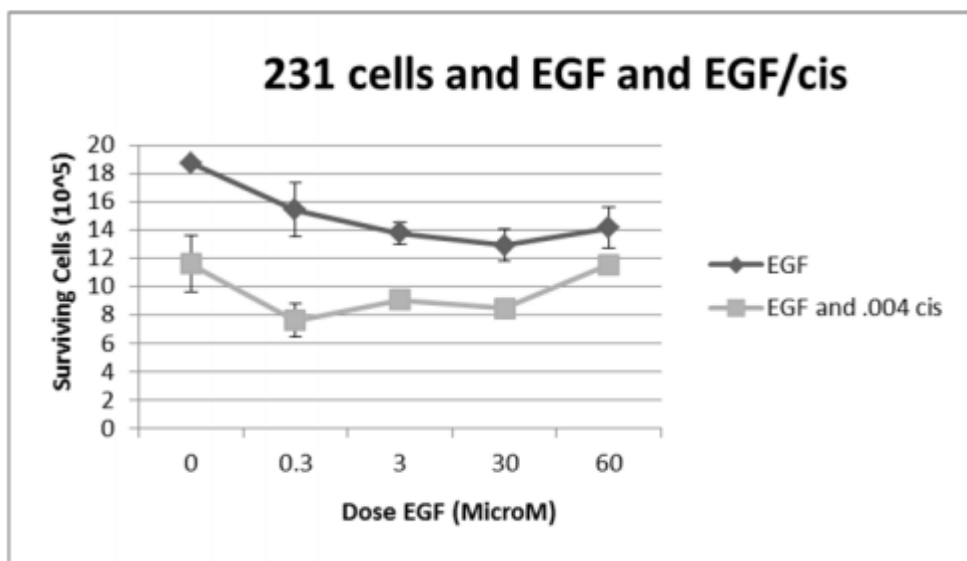


Figure 7. MDA-MB 231 cells and cisplatin treated with varying amounts of EGF.

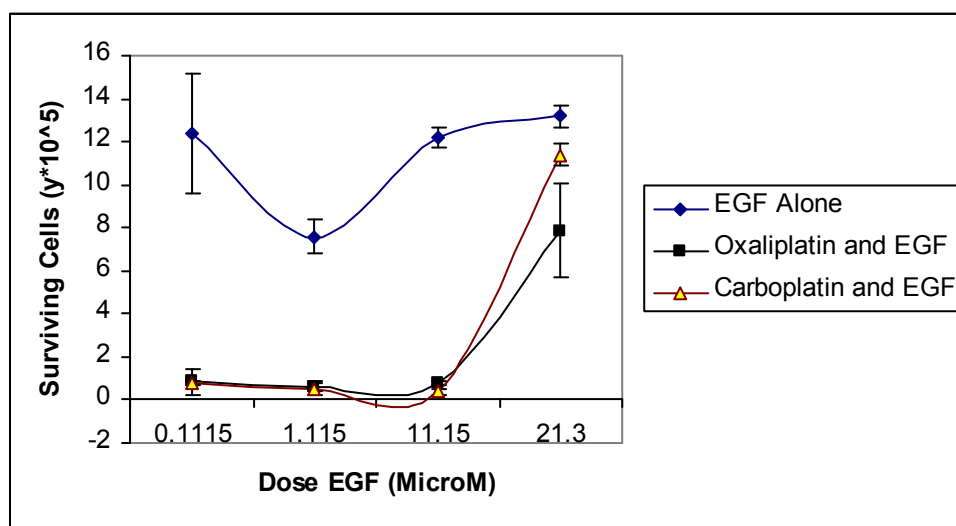


Figure 8. MDA-MB 231 cells and oxaliplatin or carboplatin treated with varying amounts of EGF

Addition of EGF-family ligands to cells treated with alkylating agents

Other ligands in the EGF family may bind to the EGFRs present on the cell surface and activate different signaling and trafficking pathways. BTC strongly stimulates receptors by binding more tightly to the receptor; at saturating concentrations, it induces efficient EGFR degradation (1). AMPHI binds to the receptor and causes internalization, but this ligand causes

the receptor to recycle back to the surface rather than undergoing lysosomal degradation. Because of their different effects on EGFR signaling and fate, the three ligands were compared for their impacts on tumor cell killing when combined with platinum-based chemotherapeutic agents.

We assumed that BTC would enhance the rate of cell death when combined with an alkylating agent to an even greater extent than EGF, due to the fact that it is most efficient in inducing EGFR degradation (1). However, my experimental results indicated that no enhancement of cell death was seen when BTC + alkylating agent was compared to alkylating agent alone (Figure 9).

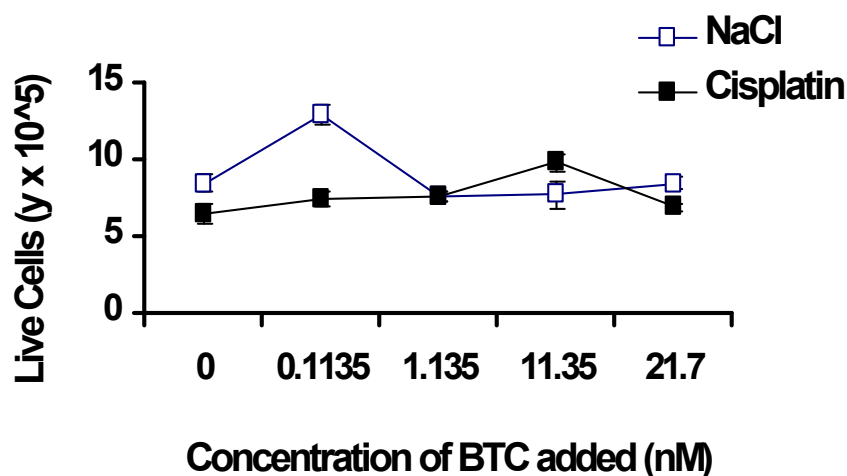


Figure 9. MDA-MB 231 cells treated with varying amounts of BTC in the presence or absence of cisplatin.

As this result was not expected, further examination of the effects of BTC on the MDA-MB 231 cells was performed. An immunofluorescence study was performed, comparing the EGFR fates in cells stimulated with EGF versus those stimulated with BTC (Figure 10). Based on the dose-response and the immunofluorescence results, the ability of a combination therapy to

enhance tumor cell killing correlated best with the therapies ability to induce the translocation of EGFR/Cbl complexes to the luminal vesicles of multivesicular endosomes (Figure 10).

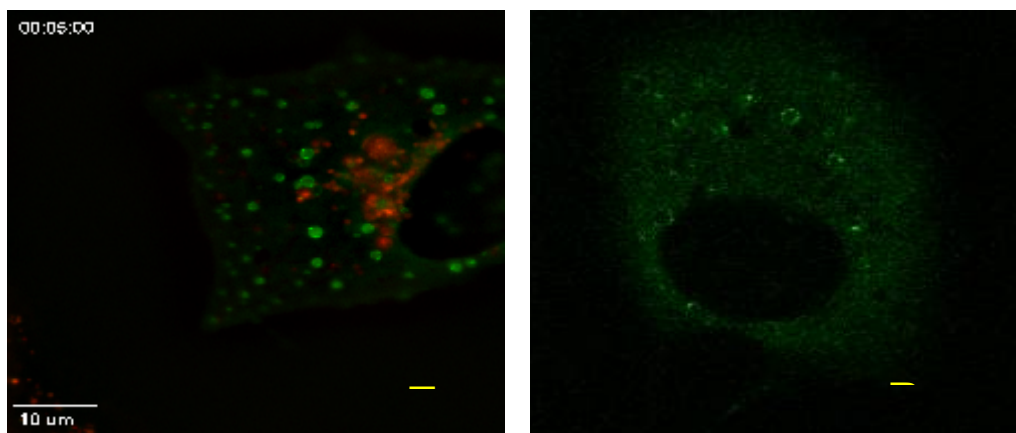


Figure 10. Although both EGF and BTC target EGFRs for degradation, they differ in their localization of EGFR/Cbl complexes at the late endosome stage. COS-7 cells were stimulated with EGF (left panel) or BTC (right panel) at 17 nM. After 25 minutes of activation, images were collected by fluorescence microscopy. The green signals correspond to GFP-Cbl wt, which is a marker for EGFR location in this experiment. EGF causes EGFR/Cbl complexes to localize to the luminal vesicles of late endosomes/multivesicular bodies. BTC maintains the complexes at the limiting membrane of these compartments (donut morphology).

Discussion

Alkylating agents cause a significant amount of cell death when acting alone. However, they cause a number of detrimental side effects, as their toxicity is not specific for cancer cells. This limits the amount of drugs that can be used to treat aggressive cancers and poses additional risks to the patient. Using EGF family ligands in combination with these compounds may enhance targeted killing of cancer cells while lowering cytotoxic effects on the body and the risk of future recurrence.

The quantity of platinum drug needed to cause a reduction in tumor cell number is well documented in the literature, both in clinical and *in vitro* settings. The calculated drug dosages

used in this study were tested extensively. Cytotoxicity curves for all three of the alkylating agents were generated. These data allowed us to identify the highest dosage that did not cause cell death. This value was then used as the reference standard for whether or not combined treatment was more effective than the alkylating agent alone. The control solution used was the vehicle in which each drug was dissolved (physiological saline for cisplatin; 10% dextrose solution for carboplatin and oxaliplatin), which allowed us to control for any effects caused by the delivery vehicle.

When EGF was combined with an alkylating agent over a six day incubation period, an enhanced rate of cell death was seen. By treating with EGF, growth was stimulated while alkylating agent acted on the cells, causing effects leading to enhanced cell death. EGF at low doses has been shown to induce receptor phosphorylation and internalization without recruitment of the Cbl E3-ubiquitin ligase or significant EGFR ubiquitination. At higher doses, EGF stimulation leads to Cbl recruitment to activated receptors, with subsequent EGFR ubiquitination and degradation in lysosomes. Our results show that an EGF dose of 3 microM is sufficient to enhance cancer cell killing by cisplatin. Because this dose is expected to not induce receptor ubiquitination, I propose that Cbl is not important to effect synergistic killing in combination with platinum drugs in our model cell system. However, we have not yet performed the ubiquitination analyses necessary to demonstrate this unequivocally.

How might the ligand EGF selectively enhance tumor cell killing by platinum drugs? Reports by other laboratories provide clues to the answer. Kroning and colleagues reported in 1995 that various malignant human cancers show increased sensitivity to platinum drugs when co-treated with EGF (6). This observation was followed by Hambek et al., who showed that prestimulation of head and neck cancer cells with EGF induced cell cycle entry by G0 phase

cells, rendering them susceptible to DNA-damaging agents (7). Both Ahsan and colleagues and Mandic and colleagues reported that many tumor cell lines that are resistant to cisplatin show increased levels of death upon prior treatment with EGF (5, 8). The work of Ahsan et al. (5) went further, revealing that cisplatin treatment increased EGFR phosphorylation at Y1045, which is the Cbl recruitment site that must be phosphorylated in order to drive activated EGF receptors through the endocytic pathway and toward lysosomes for degradation (1). Mutation of Y1045 to phenylalanine resulted in abrogation of the ability of EGF to enhance cisplatin-mediated tumor cell killing (5). Therefore, I extended the investigation of the mechanism of synergy by analyzing whether distinct EGF family ligands, known to cause differential EGFR phosphorylation and trafficking, also have differential impact on tumor cell killing platinum based drugs. Based on my results shown here, I conclude that only EGF causes an increase in tumor cell death in combination treatment with platinum based drugs. The combination treatment could have great promise clinically in enhancing tumor cell death while minimizing the dosage of alkylating agent required.

In conclusion, the combination treatment of EGF and alkylating agent enhances death in TNBC cells *in vitro*. Further trials are warranted in animal studies to test the applicability of this treatment *in vivo*.

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